# Effect of storage time and temperature on the total protein concentration and electrophoretic fractions in equine serum

Daniela Alberghina, Stefania Casella, Claudia Giannetto, Simona Marafioti, Giuseppe Piccione

## Abstract

Serum protein electrophoresis (SPE) is a technique that could be considered one of the most useful diagnostic aids available to the clinician. The effect of storage time and temperature on the total proteins and electrophoretic fractions (albumin, $\alpha_1$-, $\alpha_2$-, $\beta_1$-, $\beta_2$-, and γ-globulins) was assessed in 24 healthy horses. All samples, collected by jugular vein puncture, were centrifuged and divided into 4 aliquots. The 1st aliquot was analyzed within 3 h from collection (time 0), the 2nd was refrigerated at +4°C for 24 h, the 3rd was refrigerated at +4°C for 48 h, and the last was frozen at −20°C for 48 h. One-way repeated-measures analysis of variance (ANOVA) showed a significant effect ($P < 0.05$) of the different storage conditions on the concentrations of all the parameters studied and significant variations in the percentages of albumin, $\alpha_1$-globulins, $\alpha_2$-globulins, and γ-globulins. Compared with time 0 the total protein concentration increased significantly after 48 h at −20°C, the albumin percentage decreased after 48 h at −20°C, the $\alpha_1$-globulin percentage increased after 24 h at +4°C, the $\alpha_2$-globulin percentage increased after 48 h at +4°C and at −20°C, and the γ-globulin percentage increased after 48 h at −20°C. The results should help veterinary practitioners handle and store equine serum samples appropriately. Further investigations at different storage times and temperatures could be useful.

## Résumé

*L'électrophorèse des protéines sériques (SPE) est une technique qui pourrait être considérée comme un des outils diagnostiques les plus utiles au clinicien. L'effet du temps et de la température d'entreposage sur les protéines totales et les fractions électrophorétiques (albumine, $\alpha_1$-, $\alpha_2$-, $\beta_1$-, $\beta_2$-, et γ-globulines) a été évalué chez 24 chevaux en santé. Tous les échantillons ont été prélevés par ponction de la veine jugulaire, centrifugés et divisés en quatre aliquots. Le premier aliquot a été analysé en dedans de trois heures du moment de la collecte (temps 0), le deuxième a été réfrigéré à 4 °C pour 24 h, le troisième a été réfrigéré à 4 °C pour 48, et le dernier a été congelé à −20 °C pendant 48 h. Une analyse de variance unidirectionnelle sur des mesures répétées (ANOVA) a montré un effet significatif ($P < 0,05$) des différentes conditions d'entreposage sur les concentrations de tous les paramètres étudiés et des variations significatives dans les pourcentages d'albumine, d'$\alpha_1$-globuline, d'$\alpha_2$-globuline, et de γ-globuline. Comparativement au temps 0, la concentration de protéines totales a augmenté significativement après 48 h à −20 °C, le pourcentage d'albumine a diminué après 48 h à −20 °C, le pourcentage d'$\alpha_1$-globuline a augmenté après 24 h à 4 °C, le pourcentage d'$a_2$-globuline a augmenté après 48 h à 4 °C et à −20 °C, et le pourcentage de γ-globuline a augmenté après 48 h à −20 °C. Ces résultats devraient aider les vétérinaires praticiens à manipuler et entreposer de manière appropriée les échantillons de sérum équin. Des études ultérieures sur différents temps et températures d'entreposage seraient utiles.*

*(Traduit par Docteur Serge Messier)*

## Introduction

The serum total proteins represent the sum of numerous different proteins, many of which vary independent of each other. Since measurement of the serum total protein concentration in blood is useful to evaluate, diagnose, and monitor a variety of diseases and conditions, it is one of the most frequent routine analyses done to investigate electrolyte disorders, inflammatory or infectious diseases, colostrum intake, and tumors (1). Its routine determination is also a prerequisite of serum protein electrophoresis (SPE) (2), the most common means of fractionating serum proteins. If the results are properly interpreted, SPE could be considered one of the most useful diagnostic aids available to the clinician. This technique is used in equine medicine for diagnosis, monitoring, and prognosis of many diseases that cause changes in albumin and globulin concentrations (1). Poor performance, depression, fever, diarrhea, abdominal pain, and polyuria are clinical signs for which evaluation of the serum protein fractions is recommended (1). For diagnostic value, the measurements for all fractions must be reliable and correctly interpreted. To maximize the diagnostic value of SPE for clinical laboratories it is essential to have access to well-established relative and absolute reference values (3). Ideally reference intervals for serum or plasma parameters should be established in each laboratory; however, especially in veterinary laboratories, the establishment of reference intervals is expensive, and the ideal may not be feasible for all laboratories (4).

Standard guidelines for blood sample handling state that plasma or serum should be separated from cells as soon as possible (within 20 to 30 min) after clot formation is complete to avoid clot-induced changes in the concentration of serum analytes (5). Inadequate storage of biologic samples, a potential source of preanalytic error, may markedly affect the concentrations of biochemical variables (6,7).

Dipartimento di Scienze Veterinarie, Università degli Studi di Messina, Polo Universitario dell'Annunziata, 98168 Messina, Italy.
Address all correspondence to Prof. Giuseppe Piccione; telephone: +39 903503584; fax: +39 903503975; e-mail: giuseppe.piccione@unime.it
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**Table I. Average values and significance of differences[a] for the total protein concentration, the percentage and concentration of protein fractions, and the albumin/globulin (A/G) ratio obtained by electrophoresis of serum from 24 healthy horses analyzed under different experimental conditions**

| | Experimental condition; mean ± standard deviation | | | |
|---|---|---|---|---|
| Parameter | Within 3 h after collection (time 0) | After 24 h at +4°C | After 48 h at +4°C | After 48 h at −20°C |
| Total proteins (g/L) | 51.90 ± 3.00 | 49.70 ± 3.20 | 52.30 ± 3.00 | 67.50 ± 8.70[b] |
| Albumin (%) | 56.07 ± 5.18 | 55.08 ± 5.49 | 54.26 ± 4.22 | 53.56 ± 7.38[b] |
| Albumin (g/L) | 30.00 ± 4.00 | 30.30 ± 7.10 | 28.30 ± 2.10 | 36.20 ± 7.30[b,c,d] |
| $\alpha_1$-globulins (%) | 1.76 ± 0.39 | 2.12 ± 0.53[b] | 1.83 ± 0.33 | 1.82 ± 0.47 |
| $\alpha_1$-globulins (g/L) | 1.00 ± 0.20 | 1.10 ± 0.30 | 1.00 ± 0.20 | 1.20 ± 0.40[b,d] |
| $\alpha_2$-globulins (%) | 7.14 ± 1.46 | 7.56 ± 1.55 | 7.78 ± 1.35[b] | 8.02 ± 1.60[b] |
| $\alpha_2$-globulins (g/L) | 3.80 ± 1.10 | 4.10 ± 1.00 | 4.10 ± 0.73 | 5.40 ± 1.20[b,c,d] |
| $\beta_1$-globulins (%) | 11.98 ± 2.16 | 12.09 ± 1.89 | 12.23 ± 1.89 | 12.49 ± 2.08 |
| $\beta_1$-globulins (g/L) | 6.50 ± 1.90 | 6.50 ± 1.3 | 6.40 ± 1.10 | 8.40 ± 1.80[b,c,d] |
| $\beta_2$-globulins (%) | 6.12 ± 2.27 | 6.25 ± 2.67 | 6.18 ± 2.14 | 6.17 ± 2.40 |
| $\beta_2$-globulins (g/L) | 3.30 ± 1.20 | 3.40 ± 1.5 | 3.30 ± 1.10 | 4.20 ± 1.60[b,c,d] |
| γ-globulins (%) | 16.50 ± 3.94 | 16.50 ± 4.64 | 17.70 ± 3.15 | 17.90 ± 4.54[b] |
| γ-globulins (g/L) | 9.00 ± 3.40 | 9.60 ± 3.40 | 9.30 ± 2.00 | 12.20 ± 3.80[b,c,d] |
| A/G ratio | 1.28 ± 0.24 | 1.28 ± 0.33 | 1.20 ± 0.20 | 1.22 ± 0.46 |

[a] Significantly different ($P < 0.05$) from the values, [b]at time 0, [c]after 24 hours at +4°C, and [d]after 48 hours at +4°C by the Bonferroni multiple-comparison test.

Therefore, changes in gas composition and acid–base values (8) and clotting parameters (9) under different storage conditions have been evaluated. However, the influence of storage conditions on total proteins and electrophoretic fractions in veterinary medicine is less well-documented. In the veterinary literature the effect of storage on colorimetric serum total protein stability has been limited to dog, rat, and avian samples (6,10,11), and the effect of storage conditions on the stability of serum protein fractions has been shown for cattle (12). In horses the storage conditions for measurement of serum or plasma protein concentrations have not yet been sufficiently standardized (8,9). Considering that in equine clinical practice there is a need to carry the samples from stable to laboratory, optimal management of the preanalytic phase is important (13). Thus, the aim of this study was to evaluate the effect of short-term storage (analysis within 3 h after collection, after 24 h at +4°C, after 48 h at +4°C, and after 48 h at −20°C) on total proteins and electrophoretic fractions (albumin and $\alpha_1$-, $\alpha_2$-, $\beta_1$-, $\beta_2$-, and γ-globulins) in healthy horses.

## Materials and methods

Blood samples were obtained from 24 clinically healthy Italian Saddle Horses aged 6 to 10 y with a mean body weight of 530 ± 20 (SD) kg. The health status of the horses was checked by physical examination. The animals' vaccinations were up-to-date, and the horses were free from internal and external parasites. All housing and care conformed to the standards recommended by the Guide for the Care and Use of Laboratory Animals and Directive 86/609 CEE (14). All horses were fed standard rations, calculated to fulfill their nutritional requirements according to the specifications of the Institut national de la recherche agronomique, Paris, France (15). The standard ration comprised hay (first-cut meadow hay, sun-cured, late-cut, 8 kg/d; 6.9% crude protein on average) and a 50:50 mixture of oats and barley (approximately 3.5 kg/d). The daily ration was offered 3 times a day: at 7 am, 1 pm, and 6 pm. The mean composition values of the ration were 87% dry matter and 13% moisture. The dry matter contained 9.1% horse-digestible protein, 12.1% crude protein, 20.7% crude fiber, and 3.4% ether extract, as well as 0.80 horse feed units per 7 kg. Water was available *ad libitum*.

Blood was collected by jugular vein puncture into 10-mL Vacutainer tubes (Terumo Corporation, Japan) without anticoagulant and then centrifuged at 755 × *g* for 10 min. The obtained serum, removed with a plastic pipette and transferred into graduated Eppendorf microtubes (LP Italiana Spa, Milano, Italy), was divided into 4 aliquots and transported to the diagnostic laboratory for assessment of the total proteins and electrophoretic fractions (albumin and $\alpha_1$-, $\alpha_2$-, $\beta_1$-, $\beta_2$-, and γ-globulins). The 1st aliquot was analyzed within 3 h after collection, the 2nd was refrigerated at +4°C for 24 h, the 3rd was refrigerated at +4°C for 48 h, and the last was frozen at −20°C for 48 h. The values obtained within 3 h after collection were considered the initial concentrations, at time 0. Before the analyses the samples were thawed at room temperature (18°C to 20°C).

The total protein concentration was measured at +25°C by the biuret method with the use of commercially available reagents and an automated-analyzer ultraviolet spectrophotometer according to the procedures described by the manufacturer. This method is the most widely used colorimetric method for the determination of the total protein concentration in serum because of its simplicity, precision, and accuracy (2). The method is highly accurate for the range of total proteins likely to be found in serum (10 to 100 g/L), with linearity

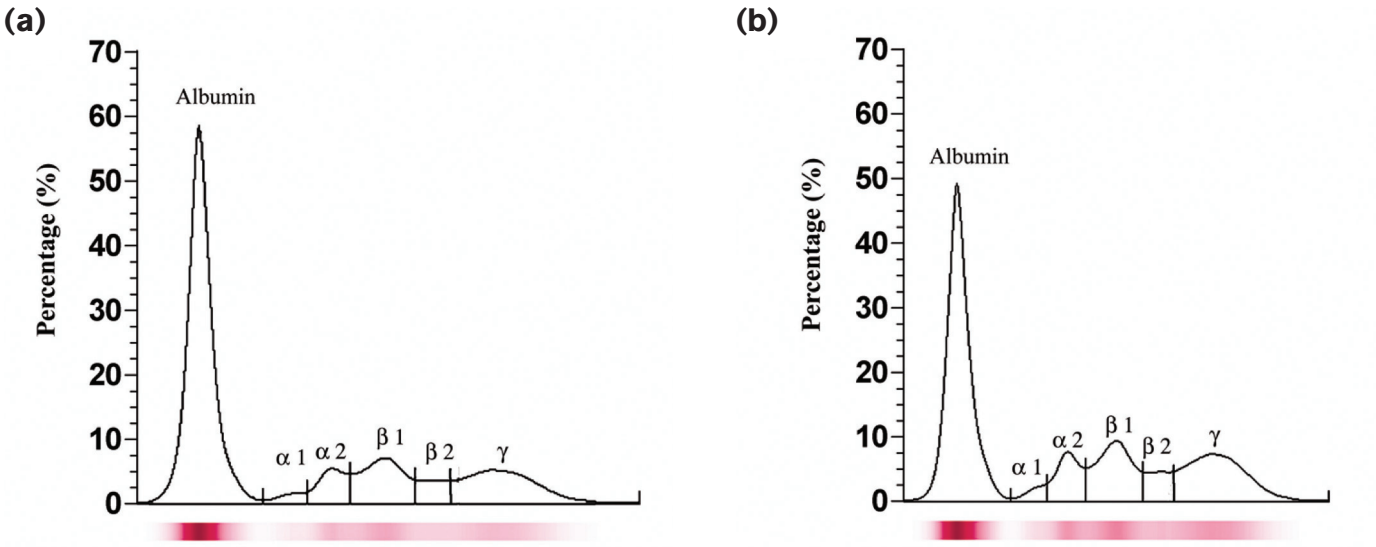


**Figure 1. Cellulose acetate electrophoretograms of serum proteins of a clinically healthy horse obtained (a) within 3 h after sample collection and (b) after storage for 48 h at −20°C. Bands were visualized by Red Ponceau S staining and densitometer scanning.**

of 0.1 g/L and sensitivity of 0.05 g/L. The amount of protein in the serum was then quantified by measuring the absorbance at 540 nm and comparing it with the absorbance of a solution containing a known concentration of a standard (bovine albumin, 52.8 g/L). The absorbance of each sample was measured in duplicate.

The protein fractions were measured by an automated system according to the procedures described by the manufacturer. For each sample, 25 µL of serum was applied to numbered wells. Each holder accommodated up to 24 samples. The proteins migrated in an electric field of 450 V for about 30 min. Electrophoresis was done on a supporting medium of cellulose acetate strips 76 × 60 mm. The buffering solution used was Tris-hippurato and distilled water (1:10). After the electrophoresis the strips were colored with Red Ponceau S for about 3 min. For destaining, a solution of orthophosphoric acid and distilled water (1:20) was applied for about 10 min. Finally, the strips were analyzed in a scanner densitometer, in which electrophoretic curves with related specific protein concentrations for each sample were displayed. The major protein fractions were divided from cathode to anode as albumin and $\alpha_1$-, $\alpha_2$-, $\beta_1$, $\beta_2$-, and γ-globulins, respectively. All samples were assayed in duplicate by the same person each time. Samples exhibited displacement parallel to the standard curve; the intra-assay coefficient of variation was $< 7\%$ and the interassay coefficient of variation $< 9\%$ for all the parameters measured. The relative concentrations of the protein fractions were determined as a percentage of the optical absorbance and as a concentration expressed in grams per liter.

All results were expressed as mean ± SD. All data were normally distributed ($P < 0.05$; Kolmogorov–Smirnov test). One-way repeated-measures analysis of variance (ANOVA) was used to determine the significance of differences related to storage conditions (time and temperature); $P$-values $< 0.05$ were considered statistically significant. The Bonferroni multiple-comparison test was used for post-hoc comparison. Data were analyzed with the Statistica 8 software package (StatSoft, Tulsa, Oklahoma, USA).

## Results

The total protein concentrations ranged from 44.60 to 57.30 g/L within 3 h after collection, from 45.00 to 55.50 g/L after 24 h at +4°C, from 44.70 to 56.60 g/L after 48 h at +4°C, and from 51.70 to 78.90 g/L after 48 h at −20°C. A significant effect ($P < 0.001$) of the experimental conditions was observed for the total protein concentration ($F_{[2,46]} = 89.48$), which significantly increased after 48 h at −20°C with respect to the previous experimental conditions (Table I). A significant effect ($P < 0.05$) of the different storage conditions was also observed for the concentrations of all the protein fractions, whereas ANOVA showed a significant effect on the percentages of only albumin ($F_{[2,46]} = 3.24$; $P < 0.05$), $\alpha_1$-globulins ($F_{[2,46]} = 4.10$; $P < 0.01$), $\alpha_2$-globulins ($F_{[2,46]} = 7.99$; $P < 0.001$), and γ-globulins ($F_{[2,46]} = 4.31$; $P < 0.01$). The Bonferroni multiple-comparison test revealed a significant effect ($P < 0.05$) of the experimental conditions on the albumin percentage, which decreased after 48 h at −20°C versus time 0, and consequently the globulin percentages increased significantly ($P < 0.05$): $\alpha_1$-globulins after 24 h at +4°C versus time 0, $\alpha_2$-globulins after 48 h at +4°C and after 48 h at −20°C versus time 0, and γ-globulins after 48 h at −20°C versus time 0.

Electrophoretograms for samples from the same horse analyzed within 3 h after collection and after 48 h at −20°C are shown in Figure 1.

## Discussion

The results showed that time and temperature have a statistically significant effect on the total proteins and their fractions in equine serum. The fractions and the A/G ratio were within the range of values obtained in horses by electrophoresis in other studies (3). As previously found for other parameters in equine samples (16), the total protein values increased after 48 h of freezing. In fact, storage of serum for up to 48 h resulted in a significant increase in total proteins only after 48 h at −20°C. Modifications of the total protein

concentration as an effect of storage conditions could be due to species-specific differences (6,10,11) or simply due to the high number of samples used in this study, which could influence the results of statistical analysis.

It is important to establish a range for serum albumin values because albumin has several important roles: a contribution to colloid oncotic pressure, maintenance of vascular permeability, modulation of coagulation, and as a carrier of many substances. In comparison with the results of other studies, in our study the albumin and γ-globulin percentages were higher (15–17) and the $\alpha_1$- and $\alpha_2$-globulin values lower (3,16–18). Thus, the results of this study suggest that the storage of equine serum for 24 h and 48 h at +4°C does not have a significant effect on total proteins, albumin, and γ-globulins but has a significant effect on α-globulins, and that the storage of serum for 48 h at −20°C considerably modifies all these parameters.

These findings demonstrate that serum protein fractions can be assessed within 48 h of blood collection when samples are stored at +4°C because refrigeration does not change the results of analysis except for α-globulins. Thus, short-term storage could influence the electrophoretic fractions and the A/G ratio. The latter is of special interest to pathologists because it allows for systematic classification of the electrophoretic profile and identification of dysproteinemias (10). However, there is a discrepancy in the veterinary literature regarding values for the physiological A/G ratio in horses (3,16–18). Equine blood samples should be transported to the diagnostic laboratory within a few hours and analyzed within 48 h at +4°C. The results found for equine species agree with those previously reported for bovine species (12) and are probably due to a different effect of temperature and time of storage on degradation of the molecular configuration of albumin with respect to globulins. Almost all proteins are glycoproteins, whereas albumin contains no carbohydrate (2). Albumin has several molecular forms and exists as a monomer and in higher aggregate states. The monomer is stable for 5 months at +8°C, whereas the dimer and the tetramer dissociate into smaller forms within a few hours (19). The decrease of albumin found in equine species agrees with results obtained in cows (12). The influence of storage on the electrophoretic distribution of globulin fractions in mammalian species of veterinary interest is still little documented.

In conclusion, storage conditions appear to have a statistically significant effect, directly correlated to temperature and time, on total proteins and their fractions in horse serum. Further investigations, at different times and temperatures, are necessary to confirm these findings and to define storage guidelines for equine samples. Reliable information about preanalytic alterations should help veterinary practitioners handle and store samples appropriately, so as to have good sample quality for correct diagnosis.

## References


1. Colahan PT, Merritt AM, Moore JN, Mayhew G. Equine Medicine and Surgery. 5th ed. Vol. II. St. Louis, Missouri: Mosby, 1999: 987–1989.
2. Kaneko JJ. Serum proteins and the dysproteinemias. In: Kaneko JJ, Harvey JW, Bruss ML, eds. Clinical Biochemistry of Domestic Animals. 5th ed. San Diego, California: Academic Press, 1997:117–137.
3. Riond B, Wenger-Riggenbach B, Hofmann-Lehmann R, Lutz H. Serum protein concentrations from clinically healthy horses determined by agarose gel electrophoresis. Vet Clin Pathol 2009; 38:73–77.
4. Stockham SL. Interpretation of equine serum biochemical profile results. Vet Clin North Am Equine Pract 1995;11:391–414.
5. Young DS, Bermes EW. Specimen collection and processing: Sources of biological variation. In: Burtis CA, Ashwood ER, eds. Tietz Textbook of Clinical Chemistry. Philadelphia, Pennsylvania: WB Saunders, 1999:42–72.
6. Cray C, Rodriguez M, Zaias J, Altman NH. Effects of storage temperature and time on clinical biochemical parameters from rat serum. J Am Assoc Lab Anim Sci 2009;48:202–204.
7. Ehsani A, Afshari A, Bahadori H, Mohri M, Seifi HA. Serum constituents analyses in dairy cows: Effects of duration and temperature of the storage of clotted blood. Res Vet Sci 2008;85:473–475.
8. Piccione G, Bertolucci C, Grasso F, Giudice E. Changes in gas composition and acid–base values of venous blood samples stored under different conditions in 4 domestic species. Vet Clin Pathol 2007;36:358–360.
9. Casella S, Giannetto C, Fazio F, et al. Assessment of prothrombin time, activated partial thromboplastin time, and fibrinogen concentration on equine plasma samples following different storage conditions. J Vet Diagn Invest 2009;21:674–678.
10. Hawkins MG, Kass PH, Zinkl JG, Tell LA. Comparison of biochemical values in serum and plasma, fresh and frozen plasma, and hemolyzed samples from orange-winged Amazon parrots *(Amazona amazonica)*. Vet Clin Pathol 2006;35:219–225.
11. Thoresen SI, Tverdal A, Havre G, Morberg H. Effects of storage and freezing temperature on clinical chemical parameters from canine serum and heparinized plasma. Vet Clin Pathol 1995;24: 129–133.
12. Tóthová C, Nagy O, Seidel H, Kovacs G. The effect of storage on the protein electrophoretic pattern in bovine serum. Iranian J Vet Sci Technol 2010;2:77–84.
13. Braun JP, Trumel C, Bézille P. Clinical biochemistry in sheep: A selected review. Small Rumin Res 2010;92:10–18.
14. Anon 1986. Directive 86/609/EEC of 24 November 1986 on the approximation of laws, regulations and administrative provisions of the Member States regarding the protection of animals used for experimental and other scientific purposes. Off J Eur Communities L 358:1–29.
15. Martin-Rosset W. L'alimentation des chevaux. Paris, France: Institut national de la recherche agronomique, 1990.
16. Carapeto V, Barrera M, Cinta Mañe R, Zaragoza MC. Serum α-globulin fraction in horses is related to changes in the acute phase proteins. J Equine Vet Sci 2006;26:120–127.
17. Cavalcante PH, Silva AC, Sakamoto S, Soto-Blanco B. Serum protein fractions in Brazilian-breed donkeys using agarose gel electrophoresis. Turk J Vet Anim Sci 2012;36:9–12.
18. Mitruka BM, Rawnsley HM. Clinical biochemistry. In: Clinical Biochemical and Hematological Reference Values in Normal Experimental Animals. New York, Masson Publishing USA, 1977: 117–245.
19. Atmeh RF, Arafa IM, Al-Khateeb M. Albumin aggregates: Hydrodynamic shape and physico-chemical properties. Jordan J Chem 2007;2:169–182.